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(54) Title: TRANSFORMATION OF PLANTS WITH NON-STRUCTURAL PLANT VIRUS GENE SEQUENCES (57) Abstract The present invention relates to a novel method whereby viral resistance is conveyed to a plant host by incorporating into the host a fragment of the viral genome associated with the replicase portion of the genome.		

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TRANSFORMATION OF PLANTS WITH NON-STRUCTURAL PLANT VIRUS GENE SEQUENCES

Since the 1986 paper of P. Powell-Abel et al [see *Science* 223:738] showing that plants transformed with and expressing the coat protein gene of Tobacco Mosaic Virus (TMV) are resistant to TMV, there have been a number of other examples of this concept which will undoubtedly have important implications for the protection of many crop species from various viral infections. To date, for example, viral coat protein-mediated resistance has been shown with alfalfa mosaic virus, tobacco rattle virus, potato virus X, cucumber mosaic virus, potyviruses, and plants transformed with both PVX and PVY coat protein.

Plant virus sequences other than that coding for the viral coat protein have been tested to determine if transformed plants can be made to exhibit resistance to post-transformation viral infection. Positive sense sequences of alfalfa mosaic virus comprising almost full length copies of RNAs 1 and 2 failed to induce resistance in transformed plants [see C. M. P. Van Dun et al, *Virology* 163:572 (1988)]; anti-sense sequences of the TMV coat protein gene did induce a low level of resistance in transformed tobacco [see P. A. Powell et al, *Proc. Nat'l Acad. Sci., USA* 86:6949 (1989)] and of potato virus X coat protein sequences [see C. Hemenway et al, *EMBO Journal* 7:1273 (1988)]; and likewise antisense RNAs from one of three regions tested (5' sequences of

RNA 1) of the cucumber mosaic virus genome gave a low level of resistance in one transformant line.

Other forms of resistance using plant transformations with DNAs prepared from satellite RNAs of plant viruses have been reported, such as the use of the satellite of cucumber mosaic virus [see B. D. Harrison et al, *Nature* 328:799 (1987)] and the concept of the ribozyme based on sequences from satellite RNAs which possess the capacity to self cleave [see J. Haseloff et al, *Nature* 334:585 (1988)].

The invention described herein represents an entirely new type of virus-induced resistance which may be transferred from one plant generation to another. The present invention discloses that transgenic plants containing a coding sequence, taken from the replicase portion of the viral genome are resistant to subsequent infection with the virus (in the description which follows, the use of the 54K coding sequence from TMV will be described in detail). In the exemplified tobacco the presence of the 54K sequence prevents the development of local chlorosis, necrosis or virus replication and any systemic development of symptoms or virus replication associated with TMV infection.

The organization of the TMV genome is fairly well understood and accepted by the scientific community. However, one aspect of the genome strategy that has not been fully elucidated is the exact nature of the replicase enzyme responsible for the synthesis of the genomic and subgenomic RNAs. While it is generally accepted that the virus codes for four

proteins, two of which are coded for by the genomic RNA, and two of which are coded for by individual subgenomic RNAs, it is not generally accepted that the virus codes for at least one other additional and separate protein.

N. D. Young et al reported [see *J. Cell Science Supplement* 7:277 (1987)] that the 5'-proximal region of the genomic RNA which encodes two coinitiated proteins, the 126K and 183K proteins, is considered to be components of the replicase. The 183K protein is generated by a read-through of the UAG stop codon of the 126K protein. The other two proteins (with known functions), the 30K protein and the coat protein are each synthesized from separate subgenomic mRNAs on which each gene is 5' proximal.

What is generally not accepted, however, is our contention that there is a separate protein, which we have labelled the 54K protein, for which there is an open reading frame in the read-through portion of the 183K gene. The principal evidence for the existence of this protein comes from the finding that there is a third subgenomic RNA in TMV infected plants, termed I₁ RNA, which initiates at nucleotide residue 3405 in the TMV genome and contains the open reading frame for a 54K protein [see M. A. Sulzinski et al, *Virology* 145:132 (1985)]. Support for its function as a mRNA and as a subgenomic RNA is derived from the observation that it is found on polyribosomes and that there is a double-stranded RNA of a size corresponding to the double-stranded version of the I₁ subgenomic RNA [see A. Zelcer et al,

Virology 113:417 (1981) and P. Palukaitis et al, *Virology* 131:533 (1983)].

More specifically, the following sequence of the region of the TMV genome containing the readthrough portion of the 183K protein gene is:

3405 3472 3495 4916
5'-GCAGGA--CAAAGACUGGUGAUAAUUUCUGAUAUG---AGUUGUAA--

-3'_. This sequence depicts the I₁ subgenomic RNA beginning at nucleotide residue 3405 (the complete sequence for the 183K protein gene may be found in (Goclet et al Proc. Natl. Acad. Sci USA 79:5818 (1982). In this sequence, the 54K open reading frame extends from nucleotide residues 3495 to 4919, and the underlined region designates the sequence used for the plant transformation more fully described in the following examples.

Unfortunately, the 54K protein has not been found in infected tissues. When antibodies to a β -galactosidase fusion protein for 432 amino acids specific to the read-through of the 126K protein expressed in *Escherichia coli* were prepared, the 54K protein in protoplast extracts could not be detected by either immunoprecipitation or Western blotting under conditions where the antibody would detect the 183K protein [see T. Saito et al, Mol. Gen. Genet. 205:82 (1986)]. Likewise, the 54K protein has not been

detectable in Western blots using antiserum made to the whole protein [see G. J. Hills et al, *Virology* 158:488 (1987)], although on occasion faint bands in the region of the gel where such a protein

would be expected have been seen. The antiserum made to the whole protein is, however, capable of precipitating the 54K protein generated from *in vitro* translation products of either TMV RNA or T7 transcripts of the 54K protein gene.

In an effort to attribute a function to the 54K protein, we have transformed tobacco with the coding sequence for this nonstructural viral protein. Unexpectedly, these transformed plants show a complete resistance to replication of the U₁ strain of TMV from which the 54K sequence was derived. This resistance was manifested when plants were inoculated with either high concentrations of virus or viral RNA.

Accordingly, a novel aspect of the present invention, is the conveyance of viral resistance to a plant which has previously undergone transformation of its normal genome with a portion of the replicase region of a viral genome, in its "sense" orientation. A more complete understanding of this aspect, as well as others of the present invention, can be had by reference to the following figures and examples.

With specific reference to the accompanying figure;

Figure 1 depicts plant expression vectors containing the TMV 54K coding sequence inserted between the CaMV 35S promoter and the nopaline synthase polyadenylation site.

More specifically, this figure shows plasmids which were derived by insertion of the TMV cDNA into either the XhoI site or the SmaI site in the polylinker region of pMON316. The numbers in these vectors refer to nucleotides in the TMV genome. The

NPTII gene confers a selectable kanamycin resistance marker to transformed plants.

EXAMPLE I

culture and maintenance of plant and virus strains

TMV strain U₁ was purified from infected *N. tabacum* cv. Turkish Samsun plants as described by A. Asselin et al [see Virology 91:173 (1978)]. Virus RNA was isolated by phenol extraction and ethanol precipitation. *N. tabacum* cv. Xanthi nn was used as a TMV-susceptible, systemic host, and *N. tabacum* cv. Xanthi nc as a local lesion host. Plants were maintained in a greenhouse or in a growth chamber with a 14 hour per 24 hour light cycle and at 24°C.

EXAMPLE II

cloning of the 54K gene

A clone of the TMV 54K gene was obtained by using a 22 base oligonucleotide primer consisting of a BamHI site linked to the 5' end of a sequence complementary to base residues 4906 to 4923 of the TMV RNA sequence. First strand DNA was synthesized by M-MLV reverse transcriptase and was rendered double stranded by sequential treatment with reverse transcriptase and Klenow relying on loop-back synthesis [see T. Maniatis et al, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY) (1982)]. The double-stranded cDNA was digested with BamHI and ligated into the BamHI site of M13mp18. The clones examined lacked the BamHI site provided by the primer. This resulted in the deletion of the 54K termination codon and the extension of the 54K protein at its C-terminus by five amino acids. The 54K insert was removed by digestion with HaeII, treated with Klenow to blunt end the 3' overhang, and finally digested with PstI. The insert was ligated into PstI/SmaI digested pBS(-) resulting in plasmid pRTT-1 which contains the TMV sequence from nucleotide residues 3472 to 4916 of the TMV RNA sequence. The orientation of the insert was such that transcription from the T7 promoter gives (+) sense transcripts as depicted in Fig. 1.

The TMV 54K sequence insert of pRTT-1 was removed by digestion with HindIII and SacI, made blunt-ended by treatment with Klenow, and ligated into either the SmaI or XhoI site of pMON316 [see S.G. Rogers et al, *Methods in Enzymology* 118:627 (1986)]. pMON316 contains a unique XhoI site in a polylinker region located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3'-untranslated region. A SmaI site is found in the polylinker region as well as within the Ti plasmid homologous region of pMON316. Plasmid pTS541A was generated by insertion of the TMV sequence into the SmaI site which resulted in the deletion of the nopaline synthase 3'-untranslated region and a portion of the Ti homology region. Insertion of the TMV sequence into the XhoI site resulted in the formation of pTS541. Clones containing the 54K sequence in either sense or antisense orientation were characterized and isolated. Each construct was transferred to *Agrobacterium tumefaciens* GV3111 carrying pTiB6S3-SE by means of a triparental mating system [see R. T. Fraley et al, *Bio/Technology* 3:629 (1985)], and transconjugates were selected by resistance to kanamycin and streptomycin.

The cDNA clone of the TMV genome RNA synthesized using a 22 base primer complementary to nucleotide residues 49006 to 4923 at the 3' end of the the 183K gene sequence resulted in the synthesis of a 54K cDNA clone with an additional 23 nucleotides at the 5' end, terminating at nucleotide 4916 as depicted in Figure 1. The presence of an intact open reading frame was verified by insertion of the TMV sequence into a T7 transcription vector; the T7 transcript was synthesized and translated in a reticulocyte lysate system. In vitro translation yielded the desired 54K product which confirmed that the AUG at position 3495 functions as an initiation codon and that the UAA codon at position 4919 functions as the stop codon. The product was verified as the desired 54K protein by immunoprecipitation using 54K antiserum.

The 54K coding sequence was subcloned into the plant expression vector pMON316 such that it is preceded by the CaMV 35S promoter and followed by the nopaline synthase 3' untranslated region as depicted in Figure 2. This construct was ultimately transferred into tobacco plants by *Agrobacterium tumefaciens*-mediated leaf disk transformation. Transformants were selected on the basis of kanamycin resistance and the production of nopaline synthase. Four transformed plants were generated with pTS541 and four other plants with pTS541A which lacks the 3' nopaline synthase untranslated region and a portion of the Ti homology region located immediately downstream from the 54K open reading frame. This deletion did not interfere with integration of the chimeric TMV 54K gene sequence into the plant

genome. Progeny seed was collected from each self-fertilized plant. Additionally, plants were transformed with the chimeric TMV gene such that 54K antisense RNAs were produced. Two independent antisense transformants were selected and regenerated into mature plants.

EXAMPLE III

plant transformation

Cut pieces of sterile, TMV susceptible, *Nicotiana tabacum* cv. Xanthi nn leaves were transformed by the modified *Agrobacterium tumefaciens* GV3111 containing the TMV 54K coding sequence as described by Horsch [see Science 227:1229 (1985)]. Transformed calli were selected on regeneration medium supplemented with kanamycin at a concentration of 300 µg/ml. Resistant calli were induced to regenerate shoots and roots, transferred to soil, and maintained in a greenhouse.

EXAMPLE IV

nucleic acid analysis

DNA was isolated from leaves of plants by a modified procedure of Murray and Thompson [see Nucleic Acids Research 8:4321 (1980)]. The DNA was digested with restriction enzymes, separated in 1.0% agarose gels, transferred to a nylon membrane, and hybridized to a ^{32}P -labeled probe specific for the TMV 54K sequence. RNA was isolated from leaf tissue and total RNA was separated in a 1.2% agarose gel containing formaldehyde and transferred to nitrocellulose filter paper. The blot was hybridized to a ^{32}P -labeled probe complementary to the 54K coding sequence.

Six of the independently transformed plants were analyzed for expression of the chimeric gene. Genomic DNA was isolated from transformed and untransformed *N. tabacum* cv Xanthi nn. BamHI digests of the genomic DNA were hybridized to a ³²P-labeled TMV 54K sequence specific probe. Hybridization to a 3.0 kb fragment verified the presence of a full length 54K coding sequence. The 54K sequence insert is 1.44 kb and another 1.59 kb is contributed by flanking vector DNA. The copy number of the 54K protein gene in transgenic plants, as determined by Southern analysis, varied from 1 to 5 copies per diploid genome between different transgenic plants; no copies of the 54K sequence were detected in nontransformed plants nor in plants transformed with pMON316 lacking the 54K sequence insert.

The TMV 54K transcripts extracted from transformed plants were also examined by Northern analysis for RNA. The expected size for the chimeric mRNA of 1.6 kb was identified in total RNA from each transgenic plant. Plants containing the integrate plasmid that lacks the 3' nopaline synthase untranslated region and the Ti homologous region also synthesize a 1.6 kb transcript. In addition, a larger transcript was synthesized which might result from the lack of the termination sequence usually contributed by the nos 3' sequence. In all plants, a number of smaller unidentified transcripts were also detected. Plants transformed with the vector alone did not produce any transcripts that hybridize with the the 54 sequence probe.

The transgenic plants were also analyzed for expression of the TMV 54K protein in accordance with Example IV. When analyzed using the Western blotting or immunoprecipitation procedures described, a 54K protein could not be detected from the 54K transgenic plants or from protoplasts prepared from 54K transgenic plants or the controls.

EXAMPLE V

immunological analyses

An antiserum to the 54K protein was made by injecting rabbits with a synthetic polypeptide representing an internal region, specifically amino acid residues 164 to 179, of the 54K protein. An in vitro translation product of the 54K T7 transcript was immunoprecipitable with the antiserum raised against the synthetic polypeptide. For Western blotting, total extracts of the transformed and untransformed plants were prepared by homogenizing leaf samples in 50 mM Tris-HCl, pH 7.5, 1% SDS, 10 mM 2-mercaptoethanol buffer; subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel; and transferred to nitrocellulose filter paper. The filter was incubated first with specific antibodies followed by gold and conjugated anti-rabbit antibodies and silver enhancement.

In studies seeking the 54K protein, 1-2 x 50 mm TMV-infected Turkish Samsun tobacco leaf strips were vacuum infiltrated with ^{35}S -methionine at a concentration of 10 $\mu\text{Ci/ml}$ in 10 mM KH_2PO_4 containing 1 mg/ml chloramphenicol. These were then incubated in dim light for 20 hrs at 25°C. Protoplasts were also labeled with ^{35}S -methionine. They were prepared from *Nicotiana tabacum* cv Xanthi NN leaves. The protoplasts (about 150,000/ml containing 5 to 10 $\mu\text{Ci/ml}$ of ^{35}S -methionine/ml) were incubated at 25°C in the light for 40 hours. These were then collected by low speed centrifugation, and lysed in 20 mM Tris-HCl, pH 7.5 buffer containing 2 mM EDTA, 0.5% SDS, 0.2% β -

mercapthethanol and 10 μ g/ml phenylmethylsulphonyl fluoride as a protease inhibitor. Leaf strips were extracted in a mortar with a similar solution, but one which did not contain the inhibitor. The extracts were then clarified by microfuge centrifugation, and the supernatants examined for the 54K protein. The presence of the 54K protein was determined by incubating the extracts of the labeled leaves or protoplasts with antisera described above; an immunoprecipitation, polyacrylamide gel, and autoradiography assays were also conducted.

This antiserum was confirmed as being very active with in vitro translation products of the 54K gene transcripts, and it could easily precipitate a 54K protein from in vitro translation products of the RNA prepared from tobacco mosaic virions containing the RNA necessary for manufacture of the 54K protein.

EXAMPLE VI

inoculation of transformed plants

RI seedlings from self-fertilized transgenic plants were routinely inoculated with either 100 μg TMV-U₁ per ml of 50 mM phosphate buffer, pH 7.2, with Celite™ added as an abrasive, or TMV-U₁ RNA at a concentration of 300 $\mu\text{g}/\text{ml}$ in pH 8.6, 50 mM Tris-phosphate buffer. Two leaves of each plant were inoculated. The volume of the inoculum was not standardized since inoculum concentration is the critical determinant as long as there is sufficient volume for adequate spread. In subsequent experiments, a closely related TMV mutant - mutant b6 as described by F. Garcia-Arenal et al, Virology 132:131 (1984) which is easier to score as a consequence of the bright yellow symptoms it elicits in the leaf. Plants were scored daily by visual observation of symptom development. In some instances, the presence of virus in inoculated plants was determined by probing leaf extracts with labeled cDNA to TMV.

In the first experiments to determine the susceptibility of the transgenic plants to infection by TMV, plants were inoculated with 50 μ g TMV-U₁ per ml. Four rooted cuttings from each of the eight independently transformed plants containing the 54K coding sequence, controls transformed with the vector alone, and several non-transformed Xanthi nn variants were inoculated. The plants were maintained in the greenhouse and monitored daily for symptom development. At 5 days post-inoculation, the transgenic controls and the non-transformed controls had clearly developed characteristic mosaic symptoms, while the transformed plants showed no sign of symptom development. No symptoms had developed on the transgenic plants by 48 days post-inoculation when the experiment was terminated. A homogenate of the inoculated and the upper leaves of those plants was used to inoculate the local lesion host, *N. tabacum* cv. Xanthi nc, to determine if a symptomless infection existed. No local lesions developed indicating the absence of detectable virus in these plants. All regenerated plants were resistant to TMV regardless of whether they were transformed with pTS541 which has the TMV sequence inserted into the complete pMON316 vector, or pTS541A which lacks the nos 3' untranslated region and the Ti homologous region. Plants transformed with the chimeric gene in the orientation which resulted in synthesis of the 54K antisense RNA were not resistant to infection with TMV. However, these plants did demonstrate a delay in system development as compared to the vector transformed control. Since this was

merely a delay in symptom development, these plants were not examined any further.

Progeny seedlings from self-fertilized transgenic plants were also analyzed for inheritability of the resistance phenomenon. R1 generation seeds were germinated on tissue culture medium containing 300 µg kanamycin per ml. Kanamycin-sensitive seedlings were considered to be those that were chlorotic and did not grow beyond the cotyledon stage. The segregation ratio of the seedlings expressing kanamycin resistance to those susceptible to kanamycin indicates that in each of the original transformants the NPTII gene was integrated at multiple loci. When seeds from self-fertilized transgenic plants were germinated on medium containing 300 µg kanamycin per ml, 95% of the seedlings emerged as being resistant to kanamycin and 5% of the seedlings became chlorotic. When transgenic seedlings were inoculated with TMV-U1 at a concentration of 100 µg/ml, 24% of these plants developed symptoms while the remaining 76 % demonstrated resistance to virus infection. Thus, the resistance to TMV segregated at approximately a 3:1 ratio (resistance:susceptible) whereas the seedlings had segregated at a ratio of approximately 19:1 with respect to the resistance to kanamycin. The large number of kanamycin resistant "escapees" makes this an unreliable means of screening progeny seedlings for expressors of the integrated chimeric TMV gene. All subsequent infection experiments were

done with the segregating population of line 541A11 derived R1 seedlings.

In an experiment to determine the level of resistance, seedlings were inoculated with varying concentrations of TMV. Resistance was observed at concentrations up to 500 μ g of TMV per ml. The resistant plants were maintained for 30 days post-inoculation without any subsequent development of symptoms. Leaf samples were taken from the inoculated plants to assay for virus replication and spread of virus. Extracts of the leaf samples were probed with cDNA prepared from purified TMV RNA. Virus could not be detected in either the inoculated leaves nor in the systemic leaves of the plants that demonstrated resistance, indicating that there is no viral replication in the resistant plants and that the resistance is absolute and not just a suppression of symptom development resulting in an asymptomatic spread of the virus throughout the plant. Transgenic plants containing the vector alone without the TMV sequence and nontransformed plants were used as controls, and virus was easily detectable in both types of control plants as well as in the progeny segregants that developed symptoms.

As a final evaluation of the resistance to viral infection of the transgenic plants, some plants were transferred immediately after inoculation to a growth chamber maintained at 31°C, to determine if the 54K-induced resistance to TMV is temperature sensitive. Of the seven inoculated plants which carry the 54K

gene sequence, five did not develop symptoms at 31°C whereas all control plants developed symptoms typical to those kept at 24°C.

In conclusion, the preceding description has demonstrated the novel aspect of the present invention that transgenic plants containing a coding sequence portion of a viral genome associated with the replicase region of the virus are resistant to infection with the virus from which the portion was initially obtained.

When compared with viral coat induced resistance, a number of advantages are present in the present invention. For example, the resistance to viral infection utilizing a replicase related code sequence as described in the present invention is not as "fragile" as coat protein-induced resistance in which resistance breaks down when high concentrations of inoculum are used. In contrast, with the present invention, complete resistance is observed in plants challenged with high concentrations of virus or viral RNA. Whereas the protection mediated by the coat proteins of TMV and A1MV can be overcome by inoculating with viral RNA, the induced resistance according to the present invention utilizing the 54K code sequence remains uncompromised when challenged with viral RNA. The level of resistance in 54K transgenic plants does not appear to be due to the level of expression: plants with only one copy of the gene sequence did not show a decrease in resistance to intact virions or viral RNA. A single copy of the TMV coat protein is also sufficient to protect the plant whereas one copy of the AiMV coat protein is not.

Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail ourselves of such changes and modifications which may be made for adapting the present invention to various usages and conditions. Accordingly, such changes and modifications are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims. The terms and expressions which have been employed in the foregoing specification are used therein as terms of description and not of limitation, and thus there is no intention, in the use of such terms and expressions, of excluding equivalents of the features shown and described, or portions thereof, it being recognized that the scope of the invention is defined and limited only by the claims which follow.

Among such modifications are, for example, the substitution of plant transformation vectors other than those specified in the examples above. For example, vectors which are within the range of substitutes or equivalents are those such as pBIN19, pBI101, pRok1, pAGS135, pARC12, PGA470, pRAL3940, and pCT1T3, among others. Although the present invention has been exemplified with TMV, other plant viruses such as cucumber mosaic, alfalfa mosaic, members of the potexvirus, bromovirus, potyvirus and luteovirus groups which also contain viral replicase regions within their genomes are also encompassed by the present

invention, as are the host plants transformed with genetic sequences related to the replicase portions of these viruses. Since it is known that similarities in sequences exist between the replicase (polymerase) regions of RNAs of many "unrelated" plant viruses [see for example, N. Habili et al., *Nucleic Acids Research* 17:9543 (1989)], including similarities between certain plant and animal RNA viruses, these are properly considered to be equivalents and therefore encompassed by the scope of the present invention.

Having thus described our invention and the manner and process of making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the art to which it pertains, or to with which it is most nearly connected, to make and use the same,

WE CLAIM:

1. A method for conveying resistance in a host to a RNA virus which comprises (1) removing a fragment of the virus RNA associated with the replicase portion of the virus genome; (2) inserting the removed fragment into a host cell in such a manner that the cell becomes transformed with the inserted fragment; and (3) causing the host cell to multiply, and differentiate into a mature host.

2. The I₁ subgenomic RNA sequence of the tobacco mosaic virus genome which is:

3405	3472	3495	4916
5'--GCAGGA---CAAAGACUGGUGAUUUUCUGAUUAUG---AGUUGUAA--			
-3'			

wherein the numerical designations indicate the position of the sequence within the TMV genome.

3. The 54K protein translated by the sequence according to Claim 2.

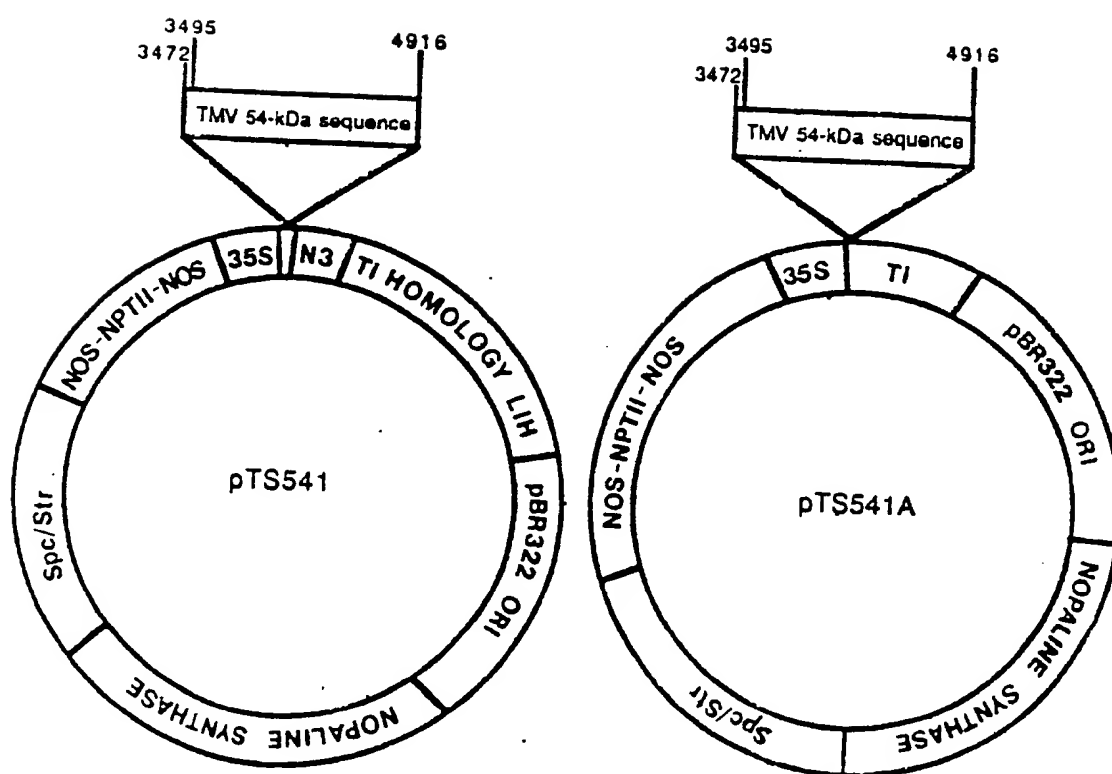
4. A transgenic plant containing a fragment of a RNA or DNA virus genome associated with the replicase portion of the viral genome.

5. A transgenic plant seed containing a fragment of a RNA virus genome associated with the replicase portion of the RNA viral genome.

6. A plant expression vector comprising a fragment of a RNA viral genome sequence associated with the replicase portion of the RNA viral genome.

1/1

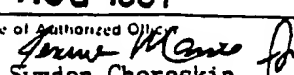
FIGURE 1



INTERNATIONAL SEARCH REPORT

International Application

PCT/US91/01631

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A01H 5/00; C12N 15/40, 9/10; C07K 15/00, 15/04 US Class: 800/205; 435/172.3, 320.1, 193; 536/27; 530/350		
II. FIELDS SEARCHED		
Minimum Documentation Searched ¹		
Classification System	Classification Symbols	
U.S.	800/205; 435/172.3, 193, 320.1, 948; 536/27; 530/350; 935/35, 64, 67	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched		
Dialog Information Services, Inc. (Onesearch Agri); USPIO Automated Patent System (File US PAT, 1971-1991). See Attachment for search terms.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ²		
Category ³	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
$\frac{X}{Y}$	Proceedings National Academy of Science, volume 79. Issued October 1982. Goelzer et al. "Nucleotide sequence of tobacco mosaic virus RNA". pages 5818-5822. see the entire document, especially Figure 1.	$\frac{2}{1-6}$
$\frac{X}{Y}$	Virology, volume 145. Issued 1985. Sulzinski et al., "Replication of tobacco mosaic virus" pages 132-140, see the entire document.	$\frac{3}{1-6}$
Y	Science, volume 232. Issued 09 May 1986, Abel et al., "Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene." pages 738-743. see the entire document.	1-6
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>⁴ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
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ISA/US		 Che Swyden Chereskin

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Nucleic Acids Research, volume 14, number 21, Issued 1986, Ishikawa, et al., "In vitro mutagenesis of the putative replicase genes of tobacco mosaic virus," pages 8291-8305, see the entire document.	1-6
X	Journal of virology, volume 21, number 2, issued February 1977, White, et al., "In vitro replication of tobacco mosaic virus RNA in tobacco callus cultures: solubilization of membrane-bound replicase and partial purification; pages 484-492. see the entire document.	3
Y	Virology, volume 81, Issued 1977, Beachy, et al., "Characterization and <u>in vitro</u> translation of the RNAs from less than full-length, virus-related, nucleoprotein rods present in tobacco mosaic virus preparations" pages 160-169. see the entire document, but especially Figure 3.	1-6
A	The EMBO Journal, volume 7, number 6, Issued 1988, Meshi, et al., "Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, Tm-1; pages 1575-1581, see the entire document.	1-6

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

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|---|---|-----|
| Y | Proceedings National Academy of Science, volume 86, Issued September 1989, Powell et al., "Protection against tobacco mosaic virus antisense RNA," pages 6949-6952, see the entire document. | 1-6 |
| A | Virology, volume 164, Issued 1988, van Dunn, et al., "Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection," pages 383-389, see the entire document. | 1-6 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹⁻³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

see attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Attachment to PCT Telephone Memorandum for PCT/US91/01631.

Reasons for Holding Lack of Unity of Invention

Group I, claims 1-2 and 4-6, drawn to sequences and plant expression vectors, method to convey resistance, and transformed plants and seeds.

Group II, claim 3, drawn to a 54 kD protein.

Inventions I and II are related as mutually exclusive species in intermediate-final product relationship. Distinctness is proven for claims in this relationship if the intermediate product is useful to make other than the final product, and the species are patentably distinct.

In the instant case, the intermediate product is deemed to be useful as a hybridization probe and the inventions are deemed distinct since there is nothing on this record to show them to be obvious variants. Should applicant traverse on the ground that the species are not distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter, fall into different statutory classes of invention and are separately classified and searched, restriction for examination purposes as indicated is proper.